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TITLE: The Distribution, Levels, and Relevance of the Interleukin-1 Family of Cytokines and Receptors in Human Breast Carcinoma-Induced Osteolysis

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14. ABSTRACT
Bone metastasis in human breast carcinoma (HBC) occurs in 83% of patients with advanced disease. HBC bone metastasis causes degeneration of the bone matrix (osteolysis), hypercalcemia, pathologic fracture, and nerve-compression syndrome. The pathophysiology of human breast carcinoma-induced osteolysis (HBC-IO) involves an increase in the number and activity of osteoclasts within the HBC metastatic lesion. We examined the expression of the IL-1 family of cytokines and receptors and IL-8 in HBC-IO using archival human samples (mean age, 52yrs; age range, 34-83yrs; no prior radiation to site) and immunohistochemistry. We observed IL-1 and IL-8 expression by HBC cells and IL-1Receptor I expression on osteoclasts. These data suggest that HBC-derived IL-1 is an important mediator of human breast cancer-induced osteolysis and supports our hypothesis: 1. IL-1 can activate osteoclastogenesis, promote osteoclast (OC) activation and osteolysis via paracrine induction of IL-1Receptors on osteoclasts. 2. IL-1 can promote tumor progression by autocrine induction and subsequent activation of IL-8. 3. IL-8 expressed by HBC cells can support tumor growth and progression by stimulating angiogenesis through IL-8 Receptors expressed on vascular endothelial cells. This study suggests that IL-1 may be an important mediator of HBC pathophysiology and therefore, a potential target for therapeutic intervention.

15. SUBJECT TERMS
Human Breast Cancer, Cytokine Networks, Bone Metastasis

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A. INTRODUCTION:

Human breast carcinoma-induced osteolysis (HBC-IO). Bone metastasis in human breast carcinoma (HBC) occurs in 83% of patients with advanced disease. Unlike most other types of cancer, breast cancer has a predilection for spreading to the bone, and bone metastasis is a major cause of increased pain, morbidity and mortality. Clinically, bone metastasis causes degeneration of the bone matrix (osteolysis), hypercalcemia, pathologic fracture, and nerve-compression syndrome. The pathophysiology of HBC-IO is not well understood. It is generally thought that HBC-IO is related to an increase in the number and activity of bone resorbing cells, the osteoclasts, at the site of the HBC metastatic lesion. These observations strongly support the involved of cell-to-cell interactions and cytokine networks in HBC-IO. IL-1 has been demonstrated in bone resorption and IL-1 has been shown to be expressed by HBC cells, however a major gap exists in our understanding of processes that occur in HBC-IO and the role of tumor-cell derived IL-1. ***Rationale/purpose:*** Since IL-1 expression has been correlated with HBC aggressiveness and IL-1 is a known activator of osteoclasts, we propose to study the levels and distribution of the IL-1 family of cytokines and receptors in HBC-IO using patient tissue samples. ***Objectives:*** 1. Demonstrate the presence, distribution and levels of the IL-1 family of cytokines and receptors [IL-1α, IL-1β, IL-1 receptor antagonist (IL-1ra), IL-1 RI, and IL-1RII] within the HBC-IO microenvironment using immunohistochemistry and ELISA, 2. Demonstrate the presence, distribution and levels of osteogenic activators/markers of osteolysis (RANKL, PTHrP and OPG) within the HBC-IO microenvironment using immunohistochemistry and ELISA, 3. Quantitate disease/lesion severity using histomorphometry (bone density, osteoclast number and distribution, tumor size and distribution), and 4. Correlate 1, 2, and 3 as described above with clinical diagnosis (primary benign bone lesions *vs.* primary malignant *vs.* bone metastasis from breast carcinoma).

B. BODY:

TASK 1. **Collect patient surgical samples.**

This study uses tissue from patients that have undergone surgical procedures related to orthopaedic oncology disease. The samples were collected under IRB except protocols and consist of archival samples or surgical discard samples.

1a. Research Accomplishments. The following type and numbers of samples have been obtained:

- Normal marrow.
  a. No bone marrow samples have been collected since these are not available as archival or surgical discard samples and in retrospect are not important to the quality or goals of this project.
- Primary benign bone tumors (giant cell tumor, non-ossifying fibroma and enchondroma).
  a. Giant cell tumors: 13 archival paraffin embedded 20 sections/ sample, 3 snap frozen.
  b. Enchondroma: 11 archival paraffin embedded 20 sections/ sample, 0 snap frozen.
- Primary malignant bone tumors (Ewing's sarcoma, malignant fibrous histiocytoma and osteogenic sarcoma).
  a. Osteosarcoma: 10 archival paraffin embedded 20 sections/ sample, 8 snap frozen
  b. Malignat fibrous histiocytoma: 6 archival paraffin embedded 20 sections/ sample, 0 snap frozen.
- Bone metastasis (breast origin). 23 archival paraffin embedded 20 sections/ sample, 2 snap frozen.

In support of obtaining human samples we have completed IRB applications and established clinical collaborations with the following institutes and individuals:

- University of Connecticut Health Center, Farmington, CT, Melinda Sanders, M.D.
- Hartford Hospital, Hartford, CT, Dr. Andrew Ricci, M.D.
- Yale University School of Medicine, New Haven CT, Dieter Lindskog, M.D.
- University of New Mexico, Robert Quinn, M.D.
- Cooperative Human Tissue Network, CHTN

1b. Problems and Solutions. This project examines the expression of cytokines in human patient samples using immunoassays such as immunohistochemistry (IHC) and ELISA. As such, we require microscope tissue sections for IHC and snap frozen pieces of tissue for ELISA. While we have been successful in obtaining sufficient samples for IHC we do not have enough snap frozen surgical discards to quantitate cytokine levels by ELISA. My collaborator, Dr. Robert Quinn, M.D., the only orthopaedic oncologist in the greater Hartford area, resigned and has established himself in New Mexico in 2004. We have re-established our research collaboration infrastructure, have an IRB (exempt) application in the final stages of approval, and expect to be obtaining samples in support of this work in the near future. I am also working with Dr. Dieter Lindskog, Yale University School of Medicine and have written an IRB (exempt) to obtain tissue. I have also established a protocol with Cooperative Human Tissue Network (CHTN), but have not found then to be a valuable resource for the specific samples I require. Despite my enthusiasm for this project, it still takes time to get the paperwork approved and get collaborators to contribute. The samples for this part of the project represent one part of the grant and other contingencies (the IL-8 studies) are in place to keep the research flowing. We have requested a no cost extension and will continue our search for snap frozen tissues.

Antigen	Isotype	Company	Product #
IL-1α	rabbit	Endogen	P420A
IL-1α	mouse	Endogen	M420A
IL-1α	mouse	Endogen	M421A
IL-1α	mouse	Biosource	AHC0912
IL-1β	rabbit	Endogen	P420B
IL-1β	mouse	Endogen	M421B
IL-1β	Goat	R + D	AF-201-
IL-1β	mouse	abcam	ab8320
IL-1ra	rabbit	abcam	ab2573
IL-1ra	rabbit	Endogen	P3001
IL-1R1	rabbit	SC	SC 687
IL-RII	mouse	R&D	MAB263
IL-8	rabbit	Endogen	P801
IL-8	Goat	R + D	AF-208-
IL-8	mouse	Endogen	M801
IL-8RA	rabbit	SC	SC-988
IL-8RB	mouse	SC	SC-7304
IL-8RB	mouse	R + D	MAB331
PTHrP	mouse	IDS	AE-0502
osteoclast	mouse	IDS	AE-4002
calcitonin receptor	mouse	Serotec	MCA2191
cathepsin K	chicken	IDS	AE-3002
(-)control	rabbit Ig	Pierce	31887
(-)control	mouse Ig	Pierce	31878

Table 1. Panel of 20/22 anti-human antibodies we have evaluated to examine HBC bone metastasis in de-calcified bone samples.

TASK 2. **Determine the distribution of the IL-1 family of cytokines and receptors in primary benign, primary malignant and breast ossous-metastatic samples.**

The major goal of this program was to determine if the pro-inflammatory cytokine, Interleukin-1 (IL-1), family of agonists, antagonists and receptors are present in the breast cancer osseous metastatic microenvironment. Furthermore, expression is to be compared to primary bone benign and malignant tumors using IHC.

2b. Research Accomplishments. Since we are exploring a new area where no published research has been done we first needed to define as establish IHC protocols IL-1α, IL-1β, IL-1 receptor antagonist (IL-1ra), IL-1Receptor I (IL-1RI) and IL-1RII using paraffin embedded samples that are de-calcified. To accomplish this we evaluated different methods of antigen retrieval using 1. citrate buffer with various heat treatments, 2. 4NHCl, and 3. DeCal Retrieval (BioGenix). Additionally we examined 20/22 different antibodies (Table 1) with these methods. The second goal of this task was to determine the expression of IL-1 by cell type and relative staining intensity. The results are summarized in table 2 and figure 1.

	IL-1β			IL-1Receptor I			IL-8		
	Number of (+) Samples	Percent (+) Cells	Staining Intensity (0-3)	Number of (+) Samples	Percent (+) Cells	Staining Intensity (0-3)	Number of (+) Samples	Percent (+) Cells	Staining Intensity (0-3)
Tumor Cells	13/16 (81%)	82%	2.3	13/16 (81%)	62%	2.0	14/17 (82%)	34%	1.3
Osteoclasts	0/16 (0%)	0%	0	15/16 (94%)	74%	2.8	0/17 (0%)	0%	0

Table 2. Immunohistochemical expression of the IL-1 family and IL-8 in HBC osteolytic lesions. No expression of IL-1RII, or IL-1Ra was observed.

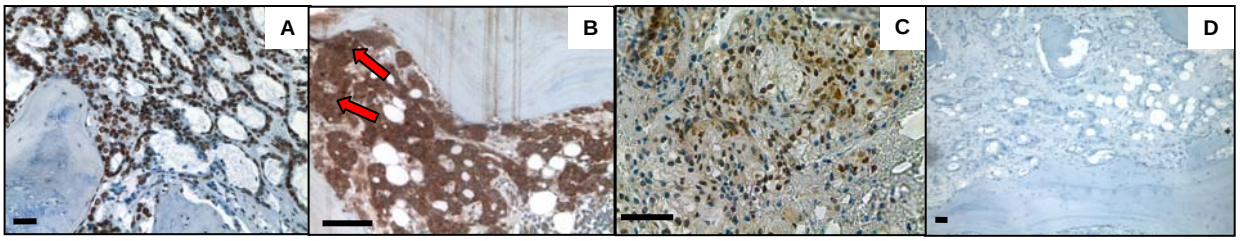


Figure 1. Immunhistochemical assay for the expression of: (a) IL-1b, (b) IL-1 RI [arrow points to staining osteoclasts], (c) IL-8 and (d) Ig negative control in human breast cancer bone metastasis. Bar = 100mm

2a Problems and Solutions. We wasted one month using the anti-osteoclast Ag antibody supplied from IDS. The technical support documents clearly support the use of this product for both formalin fixed paraffin embedded (FFPE) tissue and frozen section (OCT). The U.S. supplier assured us that it worked with FFPE and would contact technical support in the U.K. regarding this issue. The U.K. division was on "holiday" for that month and because of the importance to obtain this data for an international meeting we proceeded to trouble shoot the assay. The results from our work and later substantiate by the manufacturer, was that IDS anti-osteoclast antigen AE-4002 does not work with FFPE and the company has removed this application to it's documentation. To resolve the issue of identifying osteoclasts we have obtained anti-Calcitonin receptor (Serotec MCA2191) and Cathepsin K (IDS AE-3002). We have yet to evaluate these antibodies and would like to use them in a double-stain assay (BioCare) with IL-1 RI.

TASK 3. **Determine the distribution of cytokine and cytokine receptors that are thought to be osteogenic activators/markers of osteolysis in primary benign, primary malignant and breast ossous-metastatic samples.** This part of the study is to correlate the expression of IL-1 with receptor activator of NF-kappa B ligand (RANKL), RANKL inhibitor, osteoprotegerin (OPG) and parathyroid hormone related protein (PTHrP).
3a. Research Accomplishments. We found that the HBC tumor express PTHrP with less intensity than IL-1.
3b. Problems and Solutions. We have not evaluated the expression of RANKL or OPG. Recently RANKL was evaluated be another research group. No evidence of RANK-L expression was observed on breast cancer cells, (*personal communication*: Dr. Bhatia, University of Connecticut Health Center).

TASK 4. **Determine the levels of cytokines and receptors of the IL-1 family and osteogenic activator/markers of osteolysis.** This part of the study is to quantitate the levels of the IL-1 family of cytokines and receptors from tissue homogenates of snap frozen tissues.
4a. Research Accomplishments. Although we have obtained a small number of samples, we have not evaluated any tissue homogenates by ELISA.
4b. Problems and Solutions. We have, to date, not been able to obtain sufficient numbers of samples to complete this task. Details are described in section 1c above.

TASK 5. **Determine the relevance of cytokine and cytokine receptor expression data from immunohistochemistry and ELISA**. This part of the study is ongoing and requires completion of IL-1 expression in benign and malignant bone tumors as well as ELISA evaluation.

C. KEY RESEARCH ACCOMPLISHMENTS:

- **Established the methods and protocols for IHC evaluation of cytokines and receptors in pathological bone de-calcified FFPE tissue sections.**
- **Demonstrated the expression of IL-1b by human breast cancer cells in osseous metastatic lesions.**
- **Demonstrated the expression of IL-1R1 by human breast cells and osteoclasts in osseous metastatic lesions.**
- **Demonstrated lack of expression of the IL-1 antagonists (IL-1ra) and IL-1 RII in osseous metastatic lesions.**
- **Demonstrated expression of the pro-tumorogenic (growth and angiogenesis factor) factor, IL-8, by human breast cancer cells in osseous metastatic lesions.**

D. REPORTABLE OUTCOMES:

D1. Professional:

In April of 2004, I was promoted to Assistant Professor of Orthopaedic Surgery.

D2. Abstracts:

1. **A.G. Pantschenko,** R. Naujoks, R. H. Quinn, M. Sanders, G. Gronowicz. Cellular Cooperativity *via* Proinflammatory Cytokine Networks in the Human Breast Carcinoma Bone Metastatic Microenvironment. Skeletal Complications of Malignancy IV. April 28-30, 2005 Natcher Conference Center, NIH, Bethesda, MD (Attachment 1).
2. **A.G. Pantschenko**, R. Naujoks, R. H. Quinn, M. Sanders, G. Gronowicz. Interleukin-1 Expression in Human Breast Cancer Bone Metastasis: a Newly Recognized Pathway in Breast Cancer-induced Osteolysis. 2004. American Society for Bone and Mineral Research 26th Annual Meeting. JBMR vol 19 suppl 1 ppS227 SU094. (Attachment 2).

D3. Funding Applied for Based on Work From this Award:

1. **Title: Human Breast Cancer Bone Metastasis: Unraveling the Tumor Microenvironment using Laser Capture Microdissection and Gene Array Analysis.**
   PI: Alexander G. Pantschenko, Ph.D. **Agency:** Sidney Kimmel Foundation
   Type: Research **Period:** 7/1/05 – 6/30/07
   Compare and contrast gene expression in the osteolytic vs. non-osteolytic breast cancer osseous metastatic microenvironment.
2. **Title: Breast Cancer-derived Interleukin-1 in Osseous Metastasis.**
   PI: Alexander G. Pantschenko, Ph.D. **Agency:** NCI
   Type: KO1 Career Development **Period:** 7/1/05 – 6/30/10
   Determine the role of IL-1 in breast cancer osteolytic disease using in vitro models and a xenograft animal model with IL1 agonist, antagonist and receptor transfected human breast cancer.
3. **Title: Breast cancer osseous metastatic disease: understanding the role of proinflammatory cytokines and receptors in tumor cell modulation of the bone microenvironment.**
   PI: Alexander G. Pantschenko, Ph.D. **Agency:** Komen Fundation
   Type: Research **Period:** 4/1/05 – 3/31/06
   Understand the contribution of proinflammatory cytokines (IL-1, IL-8, & TNF) in osteoclast activation and tumor angiogenesis using xenograft model of breast cancer osseous metastasis.
4. **Title: Validation of the *In Vivo* Mouse Xenograft Models to Determine the Role of Interleukin-1 (IL-1) in Breast Cancer-induced Osteolysis.**
   PI. Alexander G. Pantschenko **Agency:** UCHC Womens' Center
   Type: Pilot **Period:** 7/1/05- 6/30/05
   Evaluate the currently used xenograft mouse model of breast cancer osseous metastasis for expression of pro-inflammatory cytokines.

5. Title: Establishing the Xenograft Model: Pro-Inflammatory Cytokines as Mediators of Tumor Progression and Osteolysis in Breast Cancer Bone Metastasis.

PI: Alexander G. Pantschenko, Ph.D. **Agency:** Dept. of Defense Breast Cancer Research Program
Type: Concept Award **Period**: 9/30/05-9/29/06

The major goal of this project is to characterize the expression and role of Interleukin-1 (IL-1) and IL-1 receptors in breast cancer bone metastasis with the murine xenograft model.

6. Title: *In Vitro* Evaluation of Therapeutic Touch on Human Breast Cancer Growth, Invasiveness and Expression of Tumor Progression Factors.

PI: Alexander G. Pantschenko, Ph.D. **Agency:** Dept. of Defense Breast Cancer Research Program
Type: Concept Award **Period**: 9/30/05-9/29/06

This project is an in vitro evaluation of the affect of therapeutic touch on human breast cancer aggressiveness.

7. Title: *In Vivo* Bioluminescent Imaging to Study Breast Cancer Osseous Metastatic Disease.

PI. Alexander G. Pantschenko **Agency:** UCHC Center for Musculoskeletal Research
Type: Pilot **Period:** 7/1/05- 6/30/05

Establish the techniques and infrastructure for bioluminescent imaging and luciferase based assays in animal models of tumor growth and metastasis.

E. CONCLUSIONS:

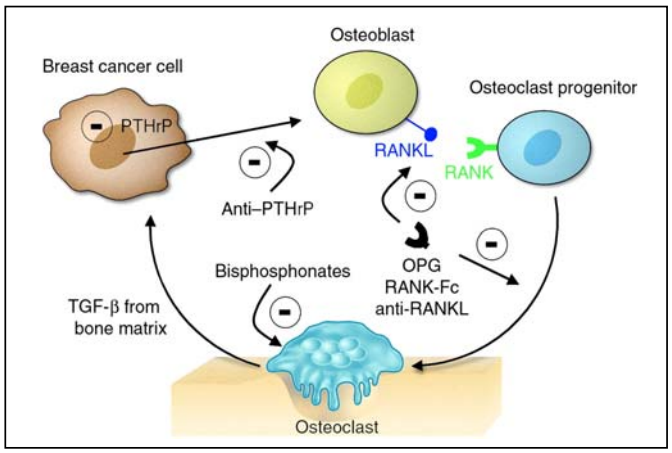


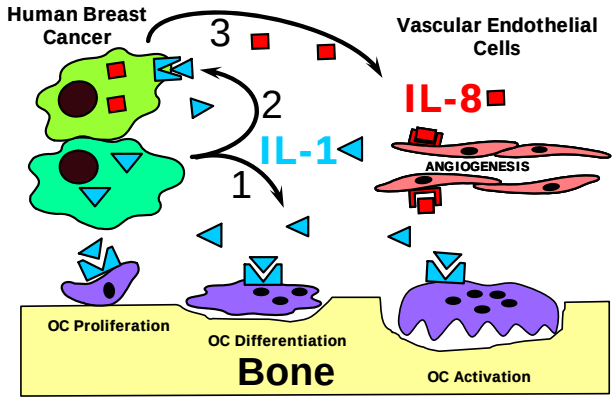
Figure 2. Proposed Role of PTHrP in Breast Cancer Osteolysis. *Adapted from T. John Martin J. Clin. Invest. 110:1399-1401 2002.*

In efforts to elucidate the mechanism of HBC-induced osteolysis a great deal of attention has been give to parathyroid hormone-related protein (PTHrP). PTHrP has been detected by immunohistochemistry in 80-90% of patients with breast cancer bone metastasis [1, 2] and increases the number of osteolytic lesions the MDA-MB-231 xenograft animal model [3]. Further animal experiments demonstrate that MDA-MB-231 cells alone or cells isolated from breast cancer samples do not express RANK-L mRNA. However, when HBC cells were co-cultured with stromal cells or osteoblasts, receptor activator of NF-kappa B-Ligand (RANK-L) mRNA was expressed and the RANK-L decoy receptor, osteoprotegerin (OPG) mRNA expression was decreased. These experiments suggest that interactions between breast cancer and stromal or osteoblastic cells induce osteoclastogenesis *in vitro* through modulating RANKL expression [4]. Therefore, PTHrP does not directly influence osteoclasts, but supports osteoclastogenesis indirectly through the upregulation of RANKL on osteoblasts. Additionally, during bone resorption, transforming growth factor beta (TGFβ) is released from the bone matrix and stimulates breast carcinoma cells to produce additional PTHrP, which in turn, perpetuates osteoclast-mediated HBC-IO (Figure 2). Although this model represents the current paradigm, it primarily derived from animal and tissue culture studies. Recent human studies dispute this mechanism for the activation of osteoclasts. Examining various osteolytic human bone tumors for RANK-L expression, Good *et. al.* were unable to show expression in breast metastasis [5]. In a larger study of 42 samples of human breast cancer bone metastasis, no evidence of RANK-L expression was observed on breast cancer cells, however osteoblasts were not evaluated (*personal communication*: Dr. Pardieb Bhatia, University of Connecticut Health Center). Clearly, other models of HBC-induced osteolysis must be explored.

We are the first to demonstrate the expression of Interleukin-1 (IL-1) by human breast carcinoma (HBC) cells and the expression of the IL-1Receptor-I on osteoclasts at the site of osteolysis in human bone samples (*appendix)* [6]. Furthermore, we have shown with cell lines that IL-1, acting through it's receptor on HBC cells,

regulates the autocrine activation of the tumor cell mitogen and angiogenesis factor, IL-8 [7, 8]. Based on our work and the literature, we hypothesize that HBC cells can directly regulate osteoclasts (and therefore osteolysis) by the IL-1 network and that IL-1 up-regulates HBC IL-8 autocrine expression. The expression of IL-8, in-turn, augments tumor angiogenesis through IL-8 Receptors on vascular endothelial cells (Figure 3). IL-1 has been demonstrated in bone resorption and IL-1 has been shown to be expressed by HBC cells, however a major gap exists in our understanding of processes that occur in HBC-Induced Osteolysis and the role of tumor-cell derived IL-1.

Figure 3. Hypothesis of the role of proinflammatory cytokines (IL-1 & IL-8) in human breast cancer-induced osteolysis based on our studies. 1. IL-1 can activate osteoclastogenesis, promote osteoclast (OC) activation and osteolysis *via* paracrine induction of IL-1Receptors on osteoclasts. 2. IL-1 can promote tumor progression by autocrine induction and subsequent activation of IL-8. 3. IL-8 expressed by HBC cells can support tumor progression by stimulating angiogenesis through IL-8Receptor expression on vascular endothelial cells.



E1. "SO WHAT".

1. This work demonstrates a previously unrecognized pathway (IL-1) in breast cancer-induced osteolysis, which is distinct and independent of the PTHrP model as described above in figure 2.
2. This work supports our hypothesis as described in figure 3 and suggests direct activation of osteoclasts by breast cancer derived IL-1.
2. This work is based on human samples and has direct relevance to human pathophysiology.
3. The PTHrP model is primary derived from animal and tissue culture studies. Our studies suggest that the currently used animal models of breast cancer osseous metastatic pathology my not be a true representation of the human disease.
4. Human breast cancer bone metastasis occurs in over 80% of patients with advanced breast cancer. Patients diagnosed with osseous-metastatic disease are considered terminal. Over the last few years the prognosis for patients with HBC bone metastasis has nearly doubled from less that a year to 18-24 months. As we are better able to treat patients, we must also hold out hope to those with advanced disease. With bone metastasis there is a significant decrease in the quality of life due to severe pain and complications associated with pathological fracture. Current palliative treatment consists of using bisphophonates, which decrease osteolysis, but has little effect on tumor and disease progression in the osseous metastatic lesion. Based on our work in orthopaedic oncology using human tissue, we developed the hypotheses that HBC-derived IL-1 mediates osteolysis by differentiation and activation of osteoclasts through the IL-1 Receptor. With this work, we have identified a new network of cytokines and receptors that contribute to disease progression. Identification of this pathway is the first step in developing new therapeutic.

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8. Pantschenko, A.G., et al., *The interleukin-1 family of cytokines and receptors in human breast cancer: implications for tumor progression.* Int J Oncol, 2003. **23**(2): p. 269-84.

G. APPENDICIES:

1. **A.G. Pantschenko,** R. Naujoks, R. H. Quinn, M. Sanders, G. Gronowicz.
   Cellular Cooperativity *via* Proinflammatory Cytokine Networks in the Human Breast Carcinoma Bone Metastatic Microenvironment. Skeletal Complications of Malignancy IV. April 28-30, 2005 Natcher Conference Center, NIH, Bethesda, MD (Attachment 1).
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ATTACHMENT 1.

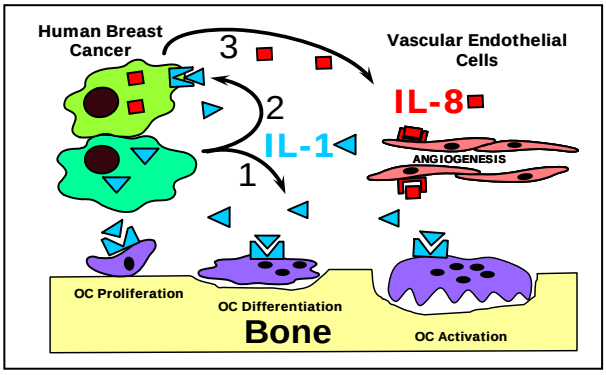
Cellular Cooperativity *via* Proinflammatory Cytokine Networks in the Human Breast Carcinoma Bone Metastatic Microenvironment.

A. G. Pantschenko[1], R. Naujoks[1], R. H. Quinn[2], M. Sanders[3], G. Gronowicz[1].
[1]Dept. of Orthopaedic Surgery, University of Connecticut Health Center, Farmington, CT, USA, [2]Dept. of Orthopaedics, University of New Mexico Health Sciences Center, Albuqerque, NM, USA, [3]Dept. of Pathology, University of Connecticut Health Center, Farmington, CT, USA. E-mail: alexgp@nso2.uchc.edu

Bone metastasis in human breast carcinoma (HBC) occurs in 83% of patients with advanced disease. Breast cancer has a predilection for spreading to the bone and bone metastasis is a major cause of increased pain, morbidity and mortality. Clinically, bone metastasis causes degeneration of the bone matrix (osteolysis), hypercalcemia, pathologic fracture, and nerve-compression syndrome. The pathophysiology of human breast carcinoma-induced osteolysis (HBC-IO) involves an increase in the number and activity of osteoclasts within the HBC metastatic lesion. These observations strongly support the involvement of cell-to-cell interactions and cytokine networks. We have recently demonstrated that the expression of the proinflammatory IL-1 family of cytokines and receptors correlates with disease severity and induction of the pro-angiogenic and mitogenic cytokine, IL-8, in HBC primary tumor. Furthermore, IL-8, a product of HBC IL-1 stimulation, has recently been shown to have a greater correlation with HBC bone metastatic potential than PTHrP in the nude mouse. Since IL-1 expression has been correlated with HBC aggressiveness and IL-1 is a known activator of osteoclasts, we examined the expression of the IL-1 family of cytokines and receptors and IL-8 in HBC-IO using archival human samples and immunohistochemistry. Histologic sections from pathological fracture resection or biopsy of HBC metastasis to bone from patients (mean age, 52yrs; age range, 34-83yrs; no prior radiation to site) were analyzed. We observed IL-1 and IL-8 expression by HBC cells and IL-1Receptor I expression on osteoclasts (Table 1). These data suggest that HBC-derived IL-1 is an important mediator of human breast cancer-induced osteolysis and supports our hypothesis: *1.* IL-1 can activate osteoclastogenesis, promote osteoclast (OC) activation and osteolysis *via* paracrine induction of IL-1Receptors on osteoclasts. *2.* IL-1 can promote tumor progression by autocrine induction and subsequent activation of IL-8. *3.* IL-8 expressed by HBC cells can support tumor growth and progression by stimulating angiogenesis through IL-8 Receptors expressed on vascular endothelial cells. This study suggests that IL-1 may be an important mediator of HBC pathophysiology and therefore, a potential target for therapeutic intervention.

	IL-1β			IL-1Receptor I			IL-8		
	Number of (+) Samples	Percent (+) Cells	Staining Intensity (0-3)	Number of (+) Samples	Percent (+) Cells	Staining Intensity (0-3)	Number of (+) Samples	Percent (+) Cells	Staining Intensity (0-3)
Tumor Cells	13/16 (81%)	82%	2.3	13/16 (81%)	62%	2.0	14/17 (82%)	34%	1.3
Osteoclasts	0/16 (0%)	0%	0	15/16 (94%)	74%	2.8	0/17 (0%)	0%	0

Table 1. Immunohistochemical expression of the IL-1 family and IL-8 in HBC osteolytic lesions. No expression of IL-1RII, or IL-1Ra was observed.



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ATTACHMENT 2.
Control/Tracking Number : 04-A-912-ASBMR
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Interleukin-1 Expression in Human Breast Cancer Bone Metastasis: a Newly Recognized Pathway in Breast Cancer-induced Osteolysis

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Bone metastasis in human breast carcinoma (HBC) occurs in 83% of patients with advanced disease. The pathophysiology of human breast carcinoma-induced osteolysis (HBC-IO) involves an increase in the number and activity of osteoclasts within the HBC metastatic lesion. These observations strongly support the involvement of cell-to-cell interactions and cytokine networks. We have recently demonstrated that the expression of the pro-inflammatory IL-1 family of cytokines and receptors correlates with disease severity and induction of pro-angiogenic and mitogenic cytokines (*e.g.* IL-8) in HBC primary tumor. Furthermore, IL-8, a product of HBC IL-1 stimulation, has recently been shown to have a greater correlation with HBC bone metastatic potential than PTHrP in the nude mouse.
Since IL-1 expression has been correlated with HBC aggressiveness and IL-1 is a known activator of osteoclasts, we examined the expression of the IL-1 family of cytokines and receptors in HBC-IO using archival human samples and immunohistochemistry. Samples from pathological fracture resection or biopsy of HBC metastasis from 16 patients (mean age, 52yrs; age range, 34-83yrs; no prior radiation to site; 14 samples from proximal femur) were obtained from the Dept. of Pathology, Hartford Hospital, Hartford, CT. and analyzed using the following antibodies; IL-1α, IL-1β, IL-1R1, IL-1R2, IL-8, CXC-R2, PTHrP, and anti-osteoclast antigen.
Thirteen of sixteen samples (81%) showed positive IL-1β tumor cell staining. Among these samples, the majority of tumor cells stained (82%). These thirteen samples were also positive for tumor cell staining of IL1-R1. Fifteen out of sixteen samples (94%) showed osteoclasts IL-1R1 staining. 14/16 showed positive staining of more than 50% of osteoclasts. 1/16 showed staining in 20-50% of cells and 1/16 sample showed no evidence of IL-1R1 staining of osteoclasts. This study supports the hypothesis that HBC tumor cell-induced osteolysis can be mediated through the HBC expression of IL-1 and the subsequent activation of osteoclasts via IL-1R1.

	IL-1β			**IL-1R1**		
	Number of (+) Samples	**Percent (+) Cells**	**Staining Intensity (0-3)**	**Number of (+) Samples**	**Percent (+) Cells**	**Staining Intensity (0-3)**
Tumor Cells	13/16 (81%)	82%	2.3	13/16 (81%)	62%	2.0
Osteoclasts	0/16 (0%)	0%	0	15/16 (94%)	74%	2.8

Author Disclosure Block: A.G. Pantschenko, None.

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DRAFT

Interleukin-1 Expression in Human Breast Cancer Bone Metastasis: a Newly Recognized Pathway in Breast Cancer-induced Osteolysis and Tumor Progression.

Running Title: Proinflammatory Cytokine Expression (IL-1 and IL-8) in Breast Cancer Osseous Metastasis.

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Conflict of Interests Statement: None.

Ethical Board Review Statement. All work described in this manuscrip was approve by the IRB committee at UCHC (#04-004) and Hartford Hospital (#03001321H).

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ABSTRACT

Bone metastasis in human breast carcinoma (HBC) is responsible for osteolysis leading to hypercalcemia, bone pain, and pathologic fracture. HBC-induced osteolysis involves an increase in the number and activity of osteoclasts, suggesting the involvement of cell-to-cell interactions and cytokine networks. We previously demonstrated the expression of the proinflammatory Interleukin (IL), IL-1 and IL-8 families of cytokines and receptors in HBC primary tumor lesions and correlated there expression to disease severity and shown that IL-1 is a potent inducer of the pro-angiogenic and mitogenic cytokine, IL-8. Since IL-1 expression has been correlated with HBC aggressiveness and IL-1 is a known activator of osteoclasts, we examined the expression of the IL-1 and IL-8 families of cytokines and receptors in HBC osseous metastatic human samples using immunohistochemistry. The majority of samples (77%) showed positive IL-1β HBC staining and IL1-R (receptor) 1 HBC staining (75%). IL-1R1 osteoclast staining was observed on all (100%) samples examined. While 75% of the samples showed IL-8 HBC staining, only 26% of HBC cells within the osseous lesion stained weakly positive. Interestingly, the IL-8 Receptor A was highly expressed in 92% of the samples staining 85% of the tumor cells and 82% of osteoclasts. IL-8RA staining was also observed on vascular endothelial cells surround both large and small blood vessels. This study supports the hypothesis that HBC tumor cell-induced osteolysis can be mediated through the HBC expression of IL-1 and the subsequent activation of osteoclasts *via* IL-1R1. Furthermore, HBC-derived IL-1 may promote angiogenesis and tumor growth by inducing the expression of IL-8. This study suggests that IL-1 may be an important mediator of HBC pathophysiology and therefore, a potential target for therapeutic intervention.

BACKGROUND

Human Breast Carcinoma-Induced Osteolysis (HBC-IO). Bone metastasis in human breast carcinoma occurs in 83% of patients with advanced disease (1), and is a major source of pain, morbidity, and mortality. Clinically, bone metastasis can cause degeneration of the bone matrix (osteolysis), pain, hypercalcemia, and pathologic fractures., The pathophysiology of HBC-IO bone destruction is related to an increase in the number and activity of bone resorbing cells, the osteoclasts, at the lesion site (2). It is also appreciated that the bone environment

contains mature and precursor osteoclasts, and osteoblasts and bone marrow stromal cells that through the deregulation of homeostasis contribute to the pathology of disease. HBC cells modulate the resident population of bone cells and uncouple the normal balance between bone resorption and bone formation resulting in a net destruction of bone. Although osteolysis and osteogenesis can be observed within the tumor-bone microenvironment, 70% of breast cancer bone metastatic lesions are osteolytic while 95% of prostate cancer lesions are purely osteoblastic (3-6). The remaining lesions are a mixture of osteolytic/osteoblastic and are observed mostly in the latter stages of the disease. The osteolytic/osteogenic criteria are a clinical definition based on radiographic examination and can be observed histologically. These observations strongly support the involved of cell-to-cell interactions and cytokine networks in HBC-IO. We hypothesize that HBC-derived IL-1 can directly support osteoclastogenesis, osteoclast activation, and therefore osteolysis. Additionally, IL-1 can indirect contribute to tumor progression by up-regulating secondary mediators such as IL-8, which in-turn support angiogenesis and tumor growth.

Interleukin-1. The cytokine interleukin 1 (IL-1) is best known as a key regulator of inflammation and immune response and has also been known by the alternate name of osteoclast activating factor. IL-1 has been appreciated as a multifunctional cytokine able to affect virtually all cell types (7-9). The IL-1 family consists of two agonists, IL-1α and IL-1β, a competitive antagonist, IL-1 receptor antagonist (IL-1ra), (10-13) and two receptors IL-1RI and IL-1RII. While IL-1α is usually intracellular and is released upon cell death/pathological condition, IL-1β is secreted out of the cell. IL-1RI is the signal transducing receptor while IL-1RII is considered an IL-1 "sink" that does not transduce signal when IL-1 is bound to it. Therefore, IL-1RI mediates IL-1 signal tranduction and IL-1RII is involved in down-regulation or inhibition of IL-1 activation.

Interleukin-1 as a Modulator of Osteoclast Activity. For over 25 years the relationship between immune activation and bone resorption has been recognized. Phytohemagglutinin stimulated human peripheral blood mononuclear cells produce a factor named osteoclast activating factor (OAF) that induces *in vitro* bone resorption (9, 14). Based on *in vitro* studies it has become clear that IL-1 can regulate osteoclastogenesis and osteoclast activation by both direct and indirect mechanisms. IL-1 has been shown to have direct activity; 1) in

the fusion of mononuclear phagocyte precursors into mature multinucleated osteoclasts (15, 16), 2) for stimulating osteoclasts to form resorption pits with mouse (16) and human cells (17), 3) as an autocrine osteoclastogenesis and bone resorption factor (18), 4) to prolong osteoclast lifespan by suppressing apoptosis (19-21), additionally, IL-1 can act with TNF to support osteoclast differentiation either independent of the receptor activator of NF-kappa B/-Ligand (RANK/RANK-L) pathway (22), or dependent on RANK-L, but independent of TNF in the mouse model (22, 23). The indirect role of IL-1 on osteoclasts has been demonstrated by the; induction of PGE2 expression in stromal/osteoblastic cells which thereby stimulate osteoclast formation (24), and indirectly stimulate resorption pit formation by modulation of osteoclasts, (25). This evidence strongly supports a role for IL-1 as a mediator of pathologic bone resorption.

Interleukin-1 and Human Breast Carcinoma (HBC). Others and we have demonstrated that the expression of the pro-inflammatory IL-1 family of cytokines and receptors correlates with HBC disease severity and expression of pro-angiogenic and mitogenic cytokines (***e.g.*** IL-8), (26-29). Interestingly, IL-8, a product of HBC IL-1 stimulation, has recently been shown to have a greater correlation with HBC bone metastatic potential than PTHrP in the nude mouse model (30-32). Therefore, IL-1 has been demonstrated as a mediator of bone resorption and IL-1 has been shown to be expressed by HBC cells, however a major gap exists in our understanding of processes that occur in HBC-induced osteolysis and the role of tumor-cell derived IL-1.

The Interleukin-8 (IL-8) and Interleukin-8 Receptors (IL-8RA and IL-8RB) Family of Cytokines and Receptors. IL-8 belongs to the CXC family of 8-10 kD proinflammatory peptides that include chemotactic cytokines, or chemokines. The CXC family (based on conserved cystine residues) of related chemokines includes GRO alpha/MGSA, GRO beta, GRO gamma, IL-8, NAP-2, ENA-78 and granulocyte chemoattractant protein-2 (33, 34). Cytokines in this family are basic heparin-binding proteins that display chemotactic activities in vitro and in vivo for a variety of cells. Additionally, these chemokines have been shown to be mitogenic for various normal and tumor cell types and activators of vascular endothelial cells, promoting angiogenesis (33, 35-42). Two distinct IL-8 receptors have been isolated, characterized and cloned, *i.e.* IL-8RA and IL-8RB (newly designated CXC-R1 and CXC-R2 respectively). IL-8 receptors are present on a variety of cell types.

Both CXC-R1 and CXC-R2 bind IL-8 with high affinity (Kd = 0.1-4.0 nM). Several other cytokines also bind to only CXC-R2 with high affinity. GRO alpha/MGSA, GRO beta, GRO gamma, and NAP-2 bind with high affinity to IL-8-R2/CXC-R2 (K_d 0.2-2.5nM) and low affinity to IL-8-R1/CXC-R1 (K_d 200-500nM), (33, 34). IL-8 binds to both receptors (CXC-R1 and CXC-R2) with high affinity.

IL-8 Cytokines and Receptors in Cancer: Although IL-8 cytokines are best known as stimulators of neutrophil and lymphocyte chemotaxis (i.e. chemokines), there is increasing evidence suggesting they play an important role in tumor angiogenesis and tumor cell proliferation. We have previously shown that in patient primary breast cancer lesions, the IL-8 levels inversely correlate with estrogen receptor and progesterone receptor levels. This suggests that IL-8 expression is associated with an aggressive breast cancer phenotype (27, 28). Previous studies have demonstrated the presence of IL-8 cytokines in a variety of neoplastic diseases, and their expression can be induced in a wide variety of tumor cells in vitro, including HBC cells. Additionally, IL-8 and/or GRO have been demonstrated in various tumor systems to be a chemotactic factor for vascular endothelial cells and tumor cells, an inducer of angiogenesis, and an autocrine/paracrine tumor cell growth factor. This data suggests that IL-1 induced tumor cell expression of IL-8 could contribute to tumor growth directly by: 1) autocrine stimulation of tumor cell activation, and 2) by paracrine activation of vascular endothelial cells resulting in tumor angiogenesis.

MATERIALS AND METHODS

Specimens.

Institutional Review Board (IRB) approved, formalin fixed, decalcified, paraffin embedded archival samples were obtained from the University of Connecticut Health Center, Department of Anatomic Pathology, Hartford Hospital, Department of Anatomic Pathology and the Cooperative Human Tissue Network (CHTN). Twenty six excisional bone samples from twenty three different patients with a diagnosis of breast cancer osseous metastasis were obtained. All samples were from female patients with a mean age of 53.8 with a range from 34 to 83 years that underwent surgical intervention between January 1995 and January 2004. The majority of samples were from the proximal femur (88%, 23/26), other sites include one sample form the proximal humerus

and two samples form the thoracic spine. Samples were selected from patients with no prior site specific radiotherapy, nor patients that had received prior chemotherapy. A Hematoxylin-Eosin (H&E) stained section from each of the samples was initially reviewed by a board certified pathology as a representation of breast cancer osseous metastatic disease.

Immunohistochemistry.

5 μm thick sections mounted on charged microscope slides were baked for 1 hour at 56^0C, deparaffinized in xylene, and rehydrated in decreasing percentages of ethanol in deionized water. Endogenous peroxidase activity was blocked by incubating 10 minutes with 3% hydrogen peroxide. Antigen retrieval was performed using citrate buffer in a steam cooker for 10 min or 4N hydrochloride acid for 10 min at 37^0C in a humid container or DeCal retrieval (BioGenex, San Ramon, CA) for 10 min at room temperature (RT). The tissue sections were washed in Tris buffered saline [50 mM Tris/HCl, 150mM NaCl (TBS)]. Nonspecific binding was blocked by incubation with Power Block (BioGenex) or Protein Block (DAKO, Carpintera, CA) for 30 minutes, RT. Initial experiments were to determine optimal antigen retrieval methods and applicability of commercially available antibodies (Table 1) for specific immunoreactivity using three concentrations within the manufacturer's suggested range. Sections were incubated with humidity at RT for 1 hour with anti-human primary antibody (Table 1) or isotype matched negative control antibody or buffer alone in TBS with 0.5% Tween-20, (TTBS) with 1% bovine serum albumin (BSA) or overnight at 2-4^0C with TBS with 1% BSA. Secondary antibody and substrate staining were done as per manufacture's instructions from the LSAB+ System, HRP (DAKO) and developed with 3,3'-diaminobenzidine (DAB) substrate. The slides were counterstained with Harris modified hematoxylin and coverslipped in Crystal/Mount aqueous-based mounting solution (Biomeda, Foster City, CA) or dehydrated and mounted in Consul-Mount (Thermo Shandon, Pittsburgh, PA).

Data Analysis and Statistical Methods.

Immuno-reactivity staining intensity is rated on a scale of 0-3, where 0 equals background staining comparable to the isotype control and 3 equals the darkest staining observed using DAB substrate. The number of positive

samples was determined by counting the number of samples with immuno-reactivity compared to the total number of samples examined. Qualitative differences were interpreted by independent observations and analyzed using the Wilcoxon signed-rank test to determine significances from 0 staining intensity, representing negative control (background) staining. For all antibodies we examined 12 -26 individual patient samples, and all values were rounded to the nearest 0.5. Some samples were excluded due to lack of adequate representation of osteoclasts (7/26) or inadequate representation of vascularity (3/12) for evaluation.

RESULTS

Interleukin-1. For those samples that had a sufficient display of osteoclasts, all samples were positive (16/16) for. No staining was observed for the IL-1 antagonist (IL-1ra), and for the mock "sink" IL-1Receptor II. IL-1 has been shown to be expressed in primary site breast cancer and IL-1, as well as TNF, have been shown to be a potent *in vitro* inducer of IL-8 protein expression. To support our hypothesis that pro-inflammatory cytokines are important in breast cancer osseous metastasis we examined the expression of the IL-1 and IL-8 family of cytokine agonists, antagonists and receptors in pathological samples of human breast carcinoma (HBC) osseous metastatic disease. All twenty–six samples showed HBC tumor cells associated with the osseous metastatic lesions. Photomicrographs of representative immunohistochemistry (IHC) experiments are shown in Figure 1, while a summary of the results are described in Table 2. Twenty of twenty-six samples (88%) showed positive IL-1β HBC tumor cell staining (Figure 1A, Table 2). Among these samples, the majority of tumor cells stained (68% range 5-90%) in a characteristic dark (strong) nuclear/perinuclear staining with modest cytoplasimic color reaction with an overall staining intensity average of 2.0/3.0. Two samples showed staining of approximately 5% of the HBC cells while the majority of samples had a more consistent 53-75% of cells staining. We also observed staining of mononuclear infiltrating cells in 2 samples that contained these cells as wells positive staining of fibroblasts associated with the desmoplastic stroma. Among the twenty-six samples, sixteen had good demonstration of osteoclasts (OCLs) suitable for evaluation by IHC and histology. To aide in the identification of osteoblast we used anti-calcitonin receptor IHC and giant cell osseous tumor as positive

control. While HBC cells also showed weak staining, OCLs stained strongly for calcitonin receptor and were further identified by their characteristic multinuclear morphology often containing vacuolated cytoplasm. For those samples that had a sufficient display of osteoclasts, all samples were positive (16/16) for osteoclast IL-1β expression representing approximately 60% of the OCL within the osseous lesion. These observations demonstrate IL-1β protein expression in HBC osseous lesions and that the major source of IL-1β are HBC cells. Central to our hypothesis is that IL-1R1 is expressed on both HBC cells to support autocrine activation and OCLs to support paracrine activation. We observed that 75% (15/20) of the samples had HBC perinuclear/cytoplasmic staining for IL-1R1 (Figure 1B and Table 2) and, as with IL-1β, two samples showed staining of approximately 5% of the HBC cells while the majority of samples had a more consistent 50-70% positive staining with an intensity of 2.5/3.0. OCLs also stained positive in 100% (16/16) of samples in a diffuse cytoplasmic pattern in 75% of the cells with an intensity of 2.0/3.0. Of the 16 samples we examined, we did not observe staining for the IL-1 antagonist (IL-1ra) nor for the mock "sink" IL-1Receptor II (reagents used are described Table 1). Since we demonstrated IL-1 agonist and receptor expression, we next examined the expression of the IL-8 family.

Interleukin-8

IL-8 is increasingly recognized as a tumor progression factor by supporting autocrine tumor cell proliferation as a growth factor and as an angiogenesis factor by stimulating vascular endothelial cell (VEC) differential in the formation of vascular support. Diffuse cytoplasmic IL-8 staining was observed in 26% of HBC cells in 75% (15/20) of samples in a patchy pattern suggestive of cell subpopulations within discrete areas of the tumor with a relatively low level of staining intensity (1.0/3.0), (Figure 1C and Table 2). Interestingly, IL-8RA was nearly ubiquitously expressed by HBC cells (85%) in nearly all of the samples examined (92%, 11/12). Furthermore, IL-8RA was observed on OCLs, as well as, large vessel (more than 3 VEC/vessel) and small vessel (4 or more VEC/vessel) VEC cells in 9/9 samples in which we identified tumor angiogenesis (Figure 1D and Table 2). These observations demonstrate the presence of IL-8 and IL-8R within the HBC osseous microenvironment.

DISCUSSION

In efforts to elucidate the mechanism of HBC-induced osteolysis, a great deal of attention has been given to parathyroid hormone-related protein (PTHrP). PTHrP has been detected by immunohistochemistry in 80-90% of patients with breast cancer bone metastasis (43-45) and increases the number of osteolytic lesions the MDA-MB-231 xenograft animal model (46). When HBC cells were co-cultured with stromal cells or osteoblasts, receptor activator of NF-kappa B-Ligand (RANK-L) mRNA was expression and the RANK-L decoy receptor, osteoprotegerin (OPG) mRNA expression was decreased. These experiments suggest that interactions between breast cancer and stromal or osteoblastic cells induce osteoclastogenesis through modulating RANKL expression (47). Therefore, PTHrP does not directly influence osteoclasts, but supports osteoclastogenesis indirectly through the upregulation of RANKL on osteoblasts and its interaction with RANK on osteoclasts. Additionally, during bone resorption, transforming growth factor beta (TGFβ) is released from the bone matrix and stimulates breast carcinoma cells to produce additional PTHrP, which in turn, perpetuates osteoclast-mediated osteolysis. Although this model represents the current paradigm, it is primarily derived from animal and tissue culture studies. Recent human studies suggest addition or alternative mechanisms for the activation of osteoclasts. Examining various osteolytic human bone tumors for RANK-L expression, Good *et. al.* were unable to show expression in breast metastasis (48). In a larger study of 43 samples of human breast cancer bone metastasis, RANK-L expression inversely correlated with breast cancer bony metastatic phenotype and no evidence of RANK-L expression was observed on breast cancer cells in the osseous metastatic site (49).

Recent studies by Larry Suva suggest that HBC tumor cell-derived IL-8 is an important mediator of osteoclastogenesis by up-regulating RANK-L expression on osteoblasts and osteolysis by directly stimulating osteoclasts (30-32). Our study of pathological samples supports the importance of IL-8 since we observe IL-8 staining on HBC cells, and IL-8RA staining of osteoclasts. We also observe IL-8RA staining of plump cuboidal osteoblasts and lining cells found along the trabecular bone (not shown). Interestingly, while we observe modest IL-8 expression, IL-8RA is strongly and nearly ubiquitously expressed on HBC cells. These observations suggest that other cytokines that bind to IL-8R (e.g. GRO) may be involved in the pathophysiology.

We have previously shown that IL-1 (both IL-1α and IL-1β) are potent inducers of IL-8 expression particular for human breast cancer cell lines that are considered most aggressive (e.g. estrogen-independent BT-20 and MDA-MB-231). IL-1 was able to increase IL-8 expression by 500-1000 fold higher than basal level in estrogen-independent cells lines while estrogen-dependent (e.g. MCF-7, T-47D and ZR-75-1) and normal mammary epithelial cells (e.g. HMEC, Hs 578 Bst, and HBL-100) show little or modest responsiveness (28). Additionally, there is 25 years of literature describing the role of IL-1 in osteoclastogenesis and osteoclast activation (9, 14-25, 50, 51). Furthermore, we have shown with human breast cancer tumor homogenates that IL-1α and IL-1β levels quantitated by ELISA directly correlate with IL-8 levels and that IL-8 inversely correlates with estrogen and progesterone receptor expression (27). These data suggests that high IL-1 and IL-8 expression is associated with aggressive human breast cancer phenotype. Therefore based on our previous and current studies, we hypothesize that HBC derived- IL-1 can directly regulate osteoclastogenesis and osteoclast activation (and therefore osteolysis) by the expression and the subsequent paracrine activation of IL-1R1 on osteoclasts. HBC derived- IL-1 can also act as an autocrine inducer of IL-8. The expression of IL-8, in-turn, augments tumor angiogenesis by the expression of IL-8 Receptors on vascular endothelial cells and may act as a tumor cell mitogen (Figure 2). IL-1 is considered a primer regulator of innate immunity and is tightly regulated on multiple levels because once IL-1 is activated, an increasing cascade of downstream mediators are subsequently released. Similarly, within the tumor microenvironment, IL-1 may act as a prime mediator. The mechanism by which IL-1 mediates its activity is *via* activation of the inhibitor-of-kappaB/nuclear factor-kappaB (IκB/NFκB) and AP-1 transcription factor pathways. NFκB (also activated by RANK/RANK-L) has been shown or implicated in the regulation of a number of pro-tumorogenic activities including: *a)* regulation of invasiveness/metastasis factors such as metalloproteinase (MMP), (52, 53), urokinase plasminogen activator (uPA), (54) and endothelial cell adhesion molecules (selectins) critical for angiogenesis (55), and *b)* a number angiogenic/mitogenic cytokines such as growth-regulated oncogene protein (GRO), (56) IL-8, vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and tumor necrosis factor (TNF) as well as the motility factor, IL-6 (39, 57-59). Based on this model, therapeutic intervention at the level of IL-1 (e.g. IL-1 antagonists) could affect not only

osteoclast activity, but also tumor progression and angiogenesis *via* IL-8/IL-8R as well as other down stream mediators activated in the NFκB and AP-1 pathways.

LEGENDS

Figure 1. Immunohistochemical expression of the pro-inflammatory IL-1 and IL-8 families of cytokines and receptors in human breast carcinoma osseous metastatic lesions.

(A. IL-1β) nuclear/perinuclear and cytoplasmic staining was observed on human breast carcinoma (HBC) cells and osteoclasts (OCL). (B. IL-1R1) cytoplasmic staining was observed by HBC cells and OCLs.

(C. IL-8) staining was mostly cytoplasmic and observed in areas of tumor tissue representative of cell subpopulations. (D. IL-8RA) staining was observed in nearly all HBC cells as well as OCLs and vascular endothelial cells (VECs). (D. Ctrl) negative control consisted of isotype matched unrelated immunoglobulin. Bar = 100μm.

Figure 2. We hypothesize that cell subpopulation cooperativity contribute to the pathophysiology of human breast cancer-induced osteolysis through the pro-inflammatory network of cytokine agonists, antagonists and receptors. *1.* IL-1 can activate osteoclastogenesis, promote osteoclast (OCL) activation and osteolysis *via* paracrine induction of IL-1Receptors on osteoclasts. *2.* IL-1 can promote tumor progression by autocrine induction and subsequent activation of IL-8. *3.* IL-8 expressed by HBC cells can support tumor progression by stimulating angiogenesis through IL-8 Receptor expression on vascular endothelial cells and may act as a breast carcinoma tumor cell growth factor.

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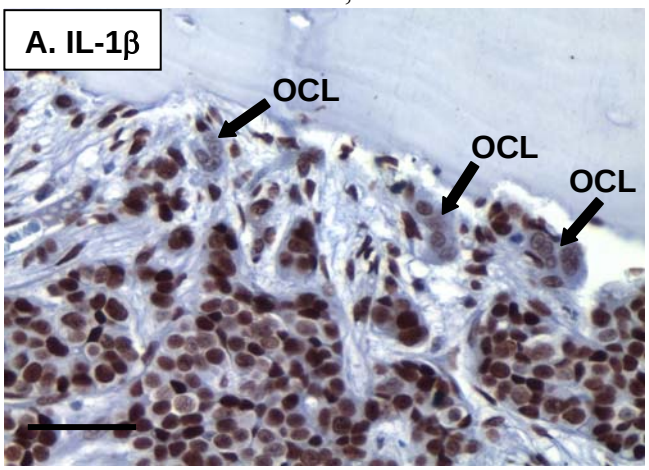
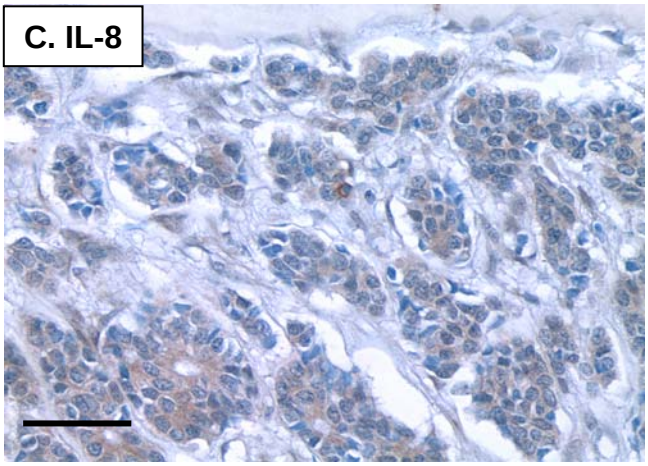
Antigen	Isotype	Company	Product #
IL-1α	rabbit	Endogen	P420A
IL-1α	mouse	Endogen	M420A
IL-1α	mouse	Endogen	M421A
IL-1α	mouse	Biosource	AHC0912
*IL-1β	rabbit	Endogen	P420B
IL-1β	mouse	Endogen	M421B
IL-1β	Goat	R + D	AF-201-
IL-1β	mouse	abcam	ab8320
IL-1ra	rabbit	abcam	ab2573
IL-1ra	rabbit	Endogen	P3001
*IL-1R1	rabbit	SC	SC 687
IL-RII	mouse	R&D	MAB263
*IL-8	rabbit	Endogen	P801
IL-8	Goat	R + D	AF-208-
IL-8	mouse	Endogen	M801
*IL-8RA	rabbit	SC	SC-988
IL-8RB	mouse	SC	SC-7304
IL-8RB	mouse	R + D	MAB331
*PTHrP	mouse	IDS	AE-0502
*Calticonin receptor	mouse	Serotec	MCA2191
(-)Control	rabbit Ig	Pierce	31887
(-)Control	mouse Ig	Pierce	31878

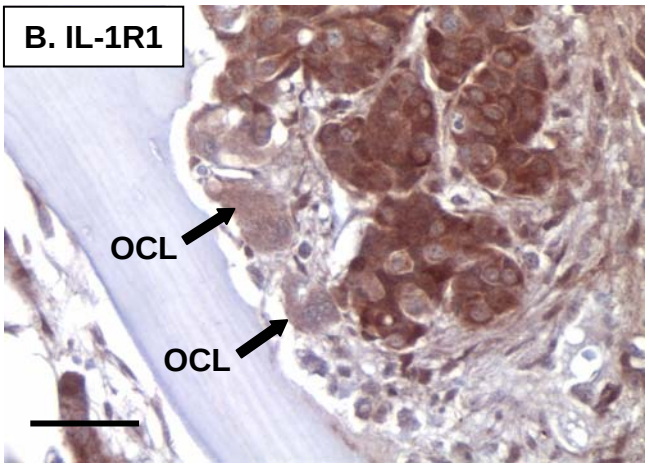
Table 1. Panel of anti-human antibodies evaluated to examine HBC bone metastasis in de-calcified paraffin embedded bone samples. * antibodies used in this study that demonstrated activity by IHC.

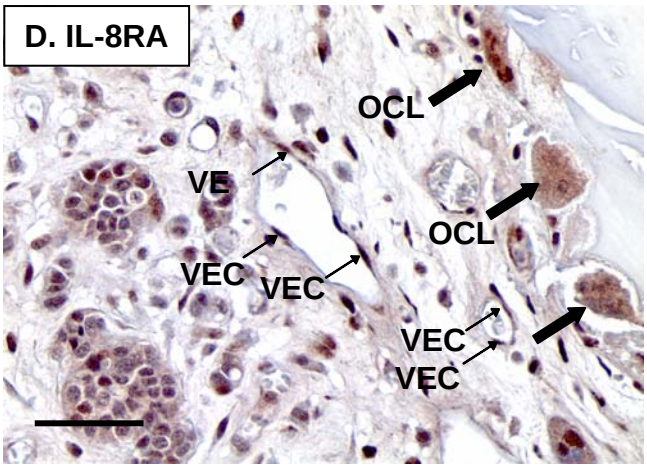
	IL-1β			IL-1 Receptor I			IL-8			IL-8 Receptor A		
	Number of (+) Samples	Percent (+) Cells	Staining Intensity (0-3)	Number of (+) Samples	Percent (+) Cells	Staining Intensity (0-3)	Number of (+) Samples	Percent (+) Cells	Staining Intensity (0-3)	Number of (+) Samples	Percent (+) Cells	Staining Intensity (0-3)
Tumor Cells	20/26 (77%)	68% (5-90%)	2.3 (0.6-3.0)	15/20 75%	58% (5-100%)	2.6 (1.5-3.0)	15/20 75%	26% (22-30%)	1.0 (0.5-1.6)	11/12 (92%)	85% (80-90%)	2.7 (2.3-3.0)
Osteoclasts	16/16 (100%)	60% (52-75%)	1.2 (0.4-1.5)	16/16 (100%)	75% (69-85%)	2.0 (0.7-2.5)	2/16	< 5%	0.4	7/9 (78%)	82% (70-89)	2.2 (1.6-3.0)

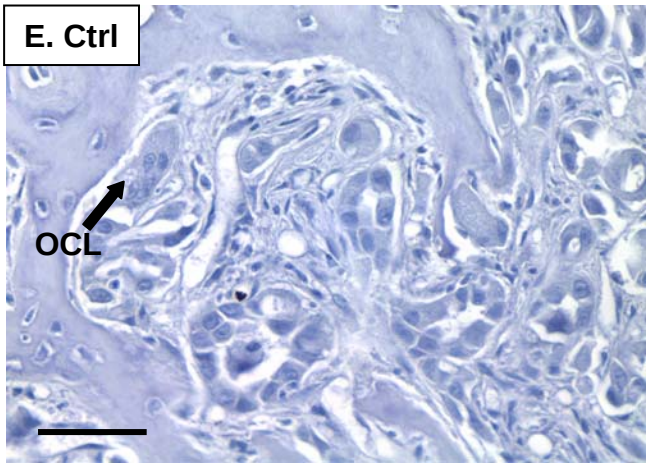
Table 2. Immunohistochemical expression of the IL-1 family and IL-8 in HBC osteolytic lesions. No expression of IL-1RII, or IL-1Ra was observed.

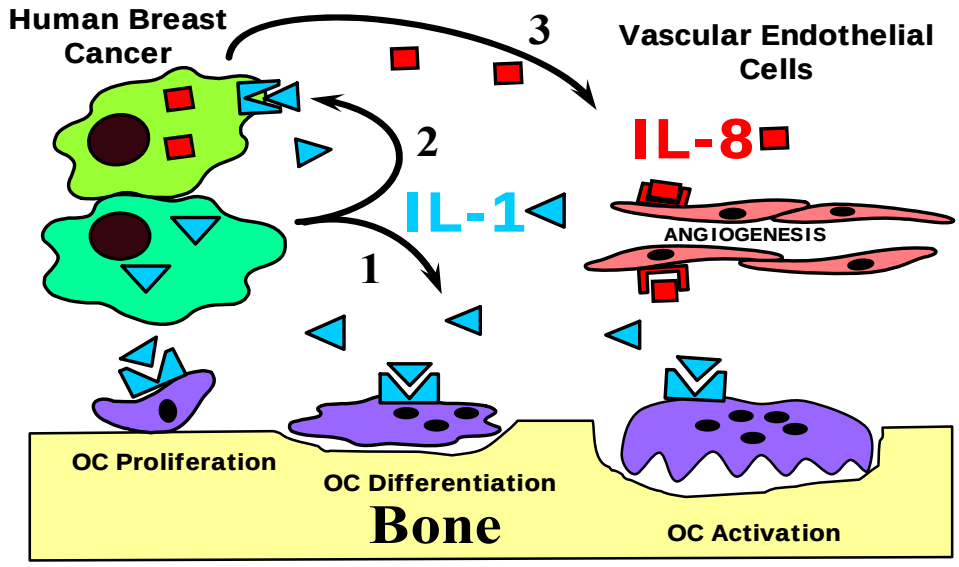

A. IL-1β
OCL
OCL
OCL


C. IL-8


B. IL-1R1
OCL
OCL


D. IL-8RA
OCL
VE
OCL
VEC
VEC
VEC
VEC


E. Ctrl
OCL


Human Breast Cancer
3
Vascular Endothelial Cells
IL-8
2
IL-1
ANGIOGENESIS
1
OC Proliferation
OC Differentiation
Bone
OC Activation